Trichohyalin: Purification from Porcine Tongue Epithelium and Characterization of the Native Protein

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Trichohyalin, a protein of M_r between 190 and 220 kDa in different species, was first demonstrated in large granules of the inner root sheath and medulla of hair follicles and may provide a matrix for keratin filaments. We have purified trichohyalin in milligram quantities from a citric acid–insoluble fraction derived from pig tongue epithelium. Trichohyalin was extracted under conditions of low ionic strength from the citric acid–insoluble fraction, separated by gel-filtration chromatography in buffer containing 1 M NaBr, and concentrated by ion-exchange chromatography in buffer containing 4 M urea. The purified material, which is soluble in buffers containing 1 M NaBr, was considered to be trichohyalin because of its characteristic molecular weight and amino acid composition and its localization to hair follicle inner root sheath and medulla by indirect immunofluorescence using antibodies against the purified protein. Immunofluorescence showed that trichohyalin is a major protein of filiform papillae of the tongue. Unlike trichohyalin from other animals examined, the porcine protein is a doublet on SDS polyacrylamide gels of 195 and 210 kDa; both bands are recognized by different antibodies, their two-dimensional peptide maps are nearly identical, and they have nearly identical isoelectric points of about 6.6. Trichohyalin has a Stokes radius of 124 A on gel filtration and a Svedberg constant of 6, consistent with an extended structure. The protein probably associates reversibly in solution, and the native protein we have isolated may be dimeric, because crosslinking of the iodinated purified protein with disuccinimidyl suberate demonstrated the presence of a dimer, which could be dissociated in the presence of high concentrations of urea. Rotary shadowing electron microscopy of the native protein showed a filamentous structure averaging 85 nm in length with a single globular-appearing end-domain. The purification of native trichohyalin provides a basis for future functional studies. *J Invest Dermatol 98:881–889, 1992*

Trichohyalin is the name given in 1903 to granules of the inner root sheath of hair follicles [1] and later to a protein extracted from the inner root sheath granules [2]. This narrow sheath, which forms a rigid insoluble tube that may guide the direction of growth of the hair fiber, has three distinct cell layers, which differentiate at different levels relative to the hair matrix. All three layers contain trichohyalin, which appears first in non-membrane bound granules that become larger in size as the cells of the internal root sheath grow further from the hair matrix and then abruptly disappear at a distinct zone (the "transformation zone") in association with the development of filaments, at which point the inner root sheath becomes birefringent [3]. Rogers found that the mature birefringent material of the inner root sheath could not be extracted with solvents used to extract keratins, such as 8 M urea with β-mercaptoethanol, but could be digested enzymatically; the digest contained citrulline [4], which was shown to be covalently bound in peptide linkage. Citrulline, an amino acid of intermediary metabolism, had not been found previously to be incorporated in protein in peptide linkage [5]. The protein containing citrulline was also found to have a high content of glutamic acid and a low content of cystine or cysteine and was therefore different from the keratin filaments of the hair shaft. Rogers also found by histochemical studies that whereas the ureasoluble trichohyalin granules contained large amounts of arginine, the mature insoluble filamentous birefringent inner root sheath contained citrulline but little arginine; both were in peptide linkage [6].

Trichohyalin was also present in the medulla of the hair fiber, where it was found to be converted to a largely nonfilamentous, amorphous or granular insoluble material with covalently bound citrulline in peptide linkage [5]. In subsequent studies the medulla, obtained from both hair and porcupine quill, has been found to have peptide-bound citrulline and also the amino acid composition of trichohyalin [5,7–9], but the structure is somewhat different from that of the inner root sheath on electron microscopy in that there are fewer filaments, which are not oriented in one direction, and more material that appears amorphous or granular [10–12]. Both the inner root sheath and the medulla were found to contain ε-(γ-glutamyl)-lysine crosslinks [9,13].

By isolating the inner root sheaths, a feat facilitated by the birefringence of the sheaths and their resistance to extraction with 8 M urea and β-mercaptoethanol, Rogers found that the citrulline-rich material contained α-helix by X-ray diffraction studies, and confirmed by direct analysis of purified inner root sheaths that the amino acid composition differed from that of keratins in its high content of citrulline, glutamic acid, and methionine and absence of cystine [14]. He proposed calling the soluble granules "arginine-trichohyalin" and the insoluble material, thought to originate from the granules, "citrulline-trichohyalin." The enzymatic activity ca-

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Abbreviations:
EDTA: ethylenediamine tetracetic acid
kDa: kilodaltons
SDS: sodium dodecyl sulfate

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pable of converting arginine to citrulline was also demonstrated [15]. Steinert later developed a technique for isolating the inner root sheath filaments [16] and characterized them biochemically [17]. Although these studies were thought at the time to support the previous notion that the filaments were composed of trichohyalin [17], it is known today that the 8–10 nm filaments like those isolated from the inner root sheath are part of a large family of filaments present in many tissues and are now defined as intermediate filaments [18]. The filaments isolated from the inner root sheath may therefore have been keratin filaments containing tightly associated, insoluble trichohyalin.

Uncertainty remains, therefore, regarding whether trichohyalin becomes an amorphous matrix for internal root sheath filaments or is itself transformed from a component of granules into filaments. The evidence for and against the presence of keratin filaments in the inner root sheath has been discussed by Fietz et al [19]. Although the deduced amino acid sequence of trichohyalin does not have the characteristic heptad repeat that results in the α-helical coiled coil of keratin filaments, structural features deduced from the cDNA sequence suggest features compatible with the formation of some type of filament [19]. In the medulla of the follicle, however, trichohyalin remains amorphous; as in the inner root sheath, arginine is converted to citrulline and the protein becomes crosslinked and insoluble, but there are fewer or no associated filaments. Evidence that the filaments of the inner root sheath are composed of a distinct group of keratins has been proposed by Heid et al [20], Ito et al [21], Lynch et al [22], and Lane et al [23]. If this is correct, trichohyalin might be associated with the keratin filaments as a crosslinking or bundling protein. O'Guin et al have studied trichohyalin with recently developed monoclonal antibodies and provided morphologic evidence that trichohyalin is an intermediate filament-associated protein [24].

Trichohyalin has been purified previously in denatured form from wool follicles by extraction with 7 M guanidine hydrochloride and gel filtration chromatography [2]. Extraction and purification under denaturing conditions permitted the production of antibody and the determination of amino acid composition and, more recently, a cDNA representing about 40% of the protein from sheep was obtained [19]. Because it has been purified previously only in denatured form, the native structure of trichohyalin has not been elucidated, and no functional studies of trichohyalin have been possible. In the course of purifying proteins from a citric acid–insoluble fraction from pig tongue epithelium (a fraction highly enriched in desmosomes), we have identified trichohyalin as a component of pig tongue and have devised a purification procedure for the undenatured protein. Using monoclonal and affinity-purified antibodies raised against the purified protein, we recently demonstrated that trichohyalin is present in isolated cells in the granular layer of human epidermis [25]. Rather than being present only in the inner root sheath of the follicle, therefore, trichohyalin is also a component of the filiform papillae of the tongue and of a limited region of the epidermis. Here we present the method devised to purify the protein in milligram quantities from pig tongue and some characterization. Unlike desmosome proteins, trichohyalin is extracted under relatively mild conditions from the insoluble fraction produced by citric acid extraction of tongue epithelium and can be purified from this fraction in quantities sufficient for characterization. The present studies lay the groundwork for future functional studies with the undenatured protein.

MATERIALS AND METHODS

Materials Superose 6, the Superose 6 HR 10/30 column, and the mono Q column were from Pharmacia (Piscataway, New Jersey). Ultrapure sucrose and urea, ^{125}I-NaI, and ^{125}I-labeled Bolton-Hunter reagent were from ICN (Cleveland, Ohio). Deoxyribonuclease (type II-S), ribonuclease (type XII-A), dithiothreitol, Tween 20, and the enzyme inhibitors leupeptin, pepstatin A, diisopropyl fluorophosphate, and phenyl methyl sulfonyl fluoride were from Sigma Chemical Co. (St. Louis, MO). Affinity-purified anti-rabbit and anti-mouse fluorophore-labeled antibodies were from Kirkegaard and Perry (Gaithersburg, Maryland). Disuccinimidyl suberate was from Pierce Chemical Co. (Rockford, IL). Other chemicals were reagent grade or better.

We raised both rabbit polyclonal antibody and a mouse monoclonal antibody against the purified protein. The antibody was affinity purified on a column containing covalently bound trichohyalin purified as described in Fig 1. Production, affinity purification, and characterization of the antibodies have been described elsewhere [25]. Affinity-purified rabbit polyclonal antibody to purified desmoplakin has been characterized previously [26].

Electrophoresis, Immunoblotting, and Peptide Mapping Polyacrylamide gel electrophoresis was performed as described [26] using 1.5-mm 3.5–17.5% gradient gels with Fairbanks' continuous buffer system [27]. Two-dimensional peptide mapping was performed according to Elder et al [28] except that both bands of the trichohyalin doublet were reduced and alkylated before being labeled with ^{125}I-NaI, and digestion was performed with chymotrypsin. The bands were lightly stained, cut out from the gel, destained, and separately iodinated in the gel for peptide mapping using Chloramine T as oxidant and 1 mCi ^{125}I-NaI. Isoelectric focussing was performed according to O'Farrell et al [29]. Protein was estimated by the heated Biuret-Folin assay [30]. Immunoblotting and quantitative immunoblotting to determine the amounts of trichohyalin present in various stages of purification were performed as described [26].

Chemical Crosslinking Crosslinking studies were performed with Bolton-Hunter–labeled protein from the final step in the purification scheme (Fig 1). The protein (50 μg) was iodinated on ice for 90 min in 10 mM sodium phosphate buffer, pH 8.0, containing 4 M urea, 1 mM ethylenediamine tetracetic acid (EDTA), 0.05% Tween 20, and 0.05 mM dithiothreitol, and the reaction was stopped with glycine (10 mM). The labeled protein (specific activity 1.1 Ci/mmol) was dialyzed overnight against the same buffer used for the iodination but with 1 mM dithiothreitol and then for 1 h against 10 mM sodium phosphate buffer, pH 8.0, containing 1 mM EDTA, 1 M NaBr, 1 mM dithiothreitol, 0.05% Tween-20, and 1 mM NaN_3. Crosslinking was performed as in the legend to Fig 6 with disuccinimidyl suberate freshly dissolved in dimethyl sulfoxide, and samples were run on agarose-polyacrylamide composite gels (0.5%–2%) [31] in the continuous buffer system of Fairbanks et al [27]. Autoradiograms were made with Dupont Cronex intensifying screens and Kodak X-OMAT AR X-ray film (Eastman Kodak, Rochester, New York) at −70°C.

Chromatography and Rate-Zonal Centrifugation Stokes radius was determined by gel filtration on a Pharmacia Superose 6 HR 10/30 column at 4°C. Purified trichohyalin or standards were analyzed using 200-μl samples containing 50 μg trichohyalin injected and eluted at 0.25 ml/min in 10 mM sodium phosphate, pH 7.4, containing 1 M NaBr, 1 mM EDTA, 0.05% Tween-20, and (for experimental samples) 1 mM dithiothreitol. Eluted proteins were detected by absorption at 280 nm. Sedimentation coefficient was determined on sucrose gradients according to Martin and Ames [32] using trichohyalin obtained from the last purification step (Fig 1). Trichohyalin (100 μg) in buffer containing 4 M urea (0.5 ml) was dialyzed for 30 min against 1 M ammonium bicarbonate containing 0.5 mM EDTA and 1 mM dithiothreitol and loaded on 13-ml linear 5–20% sucrose gradients made in 1 M ammonium bicarbonate. The gradients were centrifuged at 39,000 rpm for 21 h on a Beckman SW41 rotor and analyzed as previously described [26].

Electron Microscopy Gradients were made as above but with 15–40% glycerol in 1 M ammonium bicarbonate containing 1 mM dithiothreitol and 1 mM EDTA to prepare protein for electron microscopy. Samples were prepared for freeze-etching by a modification of the "sandwich" technique of Mould et al [33] and Loesser and Franzini-Armstrong [34]. Samples collected from glycerol gradients were diluted 1:40 to 1:160 in 1 M ammonium bicarbonate containing 1 mM dithiothreitol and 0.5 mM EDTA or in 20 mM

Tris HCl, pH 8.0, containing 1 M NaCl and 0.25 mM EDTA, and 10 μl was pipetted onto freshly cleaved mica and allowed to adhere for 2.5 min. The mica was washed three times in 0. 1 M ammonium acetate and used to make a sandwich, which was blotted dry, frozen in liquid nitrogen, and clamped onto a Balzers three-sample table under liquid nitrogen. The sandwiches were separated just before the samples were inserted into a precooled stage of a Balzers BAF 400 apparatus. The samples were etched to total dryness at −85°C and rotary shadowed at an angle of 10° with platinum, rhenium, and carbon and then at an angle of 75° with carbon [35]. The specimens were covered with a drop of 0.33% nitrocellulose isoamyl acetate, floated, washed with water, placed on grids, and examined in a Zeiss EM 10C electron microscope.

Immunofluorescence Immunofluorescence was performed on 5-μm frozen sections of tissues incubated with affinity-purified antibody to trichohyalin (5 μg/ml) or undiluted monoclonal supernatant for 16 h at 4°C and then with fluorophore-conjugated second antibody (1:100) for 1 h. Photographs were made using T-max film on a Zeiss Axioplan epifluorescence microscope with Plan Neofluor lenses.

RESULTS

Purification and Characterization of Trichohyalin Pig tongue epithelium was removed with a dermatome and extracted with citric acid–sodium citrate buffer as described [26], and trichohyalin was extracted as described in the legend to Fig 1 with low ionic strength buffer, precipitated with ammonium sulfate, and dissolved in buffer containing 1 M NaBr. The supernatant from the precipitate was resolved by gel filtration, and fractions were analyzed by SDS polyacrylamide gel electrophoresis. Fractions containing trichohyalin were pooled and loaded on a Mono Q column after equilibration with low-salt buffer containing 4 M urea and eluted with a NaBr gradient. The purification is described in detail in the legend to Fig 1, because the figure facilitates identification of each step. Figure 1 shows Coomassie-blue–stained gels of various stages in the purification scheme (*A*) and an immunoblot of the same lanes (*B*). As is shown in Table I, a thirtyfold purification yielded electrophoretically homogeneous trichohyalin on Coomassie-blue–stained gels. The purified material appeared consistently in separate preparations as a doublet of 195 and 205–210 kDa, both of which were identified by antibody to trichohyalin. To determine whether the doublet resulted from proteolysis of a single polypeptide, we shaved small slices of epidermis from pig tongue and froze these slices rapidly on dry ice, pulverized them, and extracted the frozen particles in Fairbanks' sample buffer containing SDS at 70°C. This procedure also yielded two major bands in the characteristic doublet (Fig 1, *lanes 1*), indicating that trichohyalin in pig tongue probably has two isoforms.

As noted by Rothnagel and Rogers [2], trichohyalin is very susceptible to proteolysis during purification. In an alternative procedure to the one shown in Fig 1, moderate purification was achieved with less proteolysis when the ammonium sulfate precipitate (Fig 1, *lanes 4*) was dissolved in buffered 4 M urea with inhibitors, clarified by centrifugation, dialyzed to remove excess salt, and equilibrated with buffer containing 4 M urea (10 mM sodium phosphate, pH 8.0, 1 mM EDTA, 4 M urea, 1 mM dithiothreitol, 0.05% Tween-20). Trichohyalin was then resolved directly on a Mono Q column by elution with a NaBr gradient (0–0.4 M) in the same buffer. This procedure was sometimes not adequate to remove contaminating proteins but provided samples with less degradation of the higher M_r band and a substantially higher yield (not shown). In order to determine whether the two polypeptides in the trichohyalin doublet differed substantially, we used trichohyalin obtained by this alternative purification procedure for peptide mapping. The bands were resolved on SDS 5% polyacrylamide gels and cut out of the gels, iodinated, and studied by peptide mapping [28], which indicated that the two proteins were nearly identical (Fig 2).

Although the protein was insoluble in physiologic buffers, it remained in solution in salts including NaBr, NaCl, or ammonium

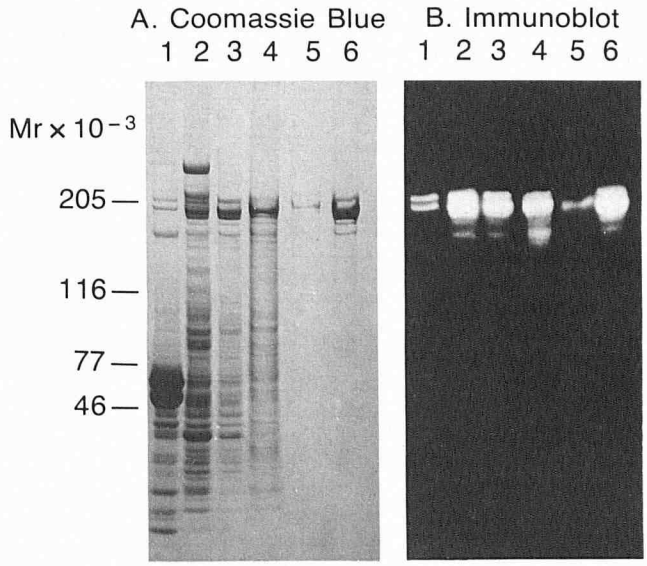

Figure 1. Purification of trichohyalin. *A*, Coomassie-blue–stained SDS polyacrylamide gels; *B*, immunoblots using affinity-purified rabbit anti-trichohyalin. Pig tongue epithelium (*lanes 1*) was processed in citric acid–sodium citrate buffer to obtain a desmosome-enriched fraction ([26], Fig 3 legend) (*lanes 2*), which was titrated to pH 8 with ice-cold 1 M Tris base and incubated with ribonuclease A and deoxyribonuclease I in the presence of 2 mM $MgCl_2$ as described previously. The protease inhibitors leupeptin (5 μg/ml), pepstatin A (5 μg/ml), phenylmethyl sulfonyl fluoride (0.2 mM), and diisopropyl fluorophosphate (1:10,000) ("inhibitors") were added at each step after titration to pH 8, and all solutions were kept on ice or at 4°C. The sample was then dialyzed extensively overnight against 0.1 mM EDTA, pH 8, containing 1 mM dithiothreitol and 0.05% Tween-20, placed in a 250-ml polycarbonate centrifuge bottle in a 37°C water bath with gentle shaking for 30 min, and centrifuged at 30,000 rpm in a Sorvall A-641 rotor for 40 min at 4°C. The supernatant (*lanes 3*) was precipitated with 55% ammonium sulfate, and the pellet collected by centrifugation (20,000 rpm, Sorvall SS-34, 30 min, 4°C) was dissolved in 10 ml of 10 mM sodium phosphate buffer, pH 8.0, containing 1 M NaBr, 1 mM EDTA, 1 mM dithiothreitol, 0.05% Tween-20, and 1 mM NaN_3, dialyzed for 1 h against the same buffer, and clarified by centrifugation at high speed (60 Ti rotor, 50,000 rpm, 60 min). The supernatant (*lanes 4*) was resolved on a Superose 6 column (2.5 × 90 cm) at a flow rate of 20 ml/h in the same buffer and fractions containing trichohyalin as determined by SDS polyacrylamide gel electrophoresis were pooled (40 ml, lanes 5) and dialyzed against 10 mM sodium phosphate buffer, pH 8.0, containing 4 M urea, 1 mM EDTA, 1 mM dithiothreitol, 0.05% Tween-20, and 1 mM NaN_3. The sample was loaded on a Mono Q column (Pharmacia, Piscataway, New Jersey) at 0.5 ml/min, which was washed with the same buffer for 1 h and eluted with a 0–0.4 M NaBr gradient (0.5 ml/min, 0–100% B in 60 min). Fractions containing trichohyalin by SDS polyacrylamide gel electrophoresis were pooled (*lanes 6*).

bicarbonate at concentrations of 0.8 M or higher and dithiothreitol (1 mM) and buffered to a pH of 7–8. High-performance liquid chromatography gel filtration in buffer containing 1 M NaBr indicated a Stokes radius of 124 Å in comparison with standards (ovalbumin [28 Å], catalase [52 Å], thyroglobulin [85 Å], and desmoplakin II [90 Å]; data not shown). Because the Superose 6 HPLC column gave poor resolution of trichohyalin in relation to standards with larger Stokes radii, results were corroborated using Fractogel TSK, HW-55 (MCB Reagents, Gibbstown, New Jersey), which better resolves molecules with large Stokes radii (greater than 100 Å); on this column trichohyalin eluted in an intermediate position between desmoplakin I (164 Å) and desmoplakin II (90 Å), confirming the result with the Superose 6 column (data not shown). Rate-zonal centrifugation performed on sucrose gradients in buffers containing 1 M NaBr showed that trichohyalin has a Svedberg constant of 6 (standards were cytochrome c ($s_{20,w} = 1.75$), bovine

Table I. Purification of Trichohyalin

Fraction	Protein[a] (mg)	Trichohyalin[b] (mg)	Purification (-fold)	Yield (%)
Citrate insoluble	839	27.9	1	100
37°C low salt supernatant	180	22.2	3.7	80
1 M NaBr soluble	25.5	7.2	8.5	26
Gel filtration eluate	5.10	2.8	16.5	10
Mono Q eluate	0.88	0.88	30	3

[a] Determined by heated Biuret-Folin assay [30].
[b] Determined by quantitative immunoblotting.

serum albumin (4.6), aldolase (7.3), and catalase (11.3); data not shown). The large Stokes radius and low sedimentation constant suggested an extended rather than globular configuration. Isoelectric focusing of the purified protein showed that it is slightly acidic, having an isoelectric point of about 6.6 (Fig 3). The physical data are summarized in Table II. Analysis of the amino acid composition of trichohyalin revealed unusually high proportions of glutamine/glutamate (approximately 33%) and arginine (approximately 31%) (Table III), similar to previously reported results [19] but showing an even higher proportion of arginine.

Demonstration of Trichohyalin in Filiform Papillae of Tongue Both polyclonal and monoclonal antibodies to trichohyalin demonstrated the characteristic distribution of trichohyalin in the inner root sheath and medulla of mouse vibrissae and of human hair [25]. Studies of pig tongue epithelium, from which trichohyalin was purified, showed that the protein was present exclusively in the filiform papillae; other types of papillae did not stain. Whereas desmoplakin staining of the cellular periphery was present throughout the epithelium of the tongue, both in the filiform papillae and in the interpapillary epidermis (Fig 4*A*), trichohyalin staining was limited to the papillae (Fig 4*B*). Trichohyalin appeared as densely stained granules in the lower parts of the papillae, and the granules were larger in the upper, more differentiated region of the papillae (Fig 5). Still higher in the papillae the granules were no longer visible, and staining of cells was more homogeneous. The transition from smaller to larger granules and then to homogeneous staining in the filiform papillae, as has been described in the follicular inner root sheath, suggests that the protein may undergo the same transformation process in the tongue as in the inner root sheath, involving crosslinking and conversion of arginine to citrulline (summarized by Rothnagel and Rogers [2]). Both monoclonal and polyclonal antibodies identified trichohyalin in lingual epithelium of other species, including mouse, dog, cow, sheep, and human (not shown), indicating that the epitopes are substantially conserved and that the protein is likely to be widely distributed in mammals. The protein from snap-frozen tongue in these species, unlike that in the pig, appeared to be a single polypeptide rather than a doublet on our gels by both Coomassie blue staining and immunoblotting.

Crosslinking Studies In order to determine whether purified trichohyalin is monomeric in solution, we performed crosslinking studies of the purified protein. The radioactively labeled protein was

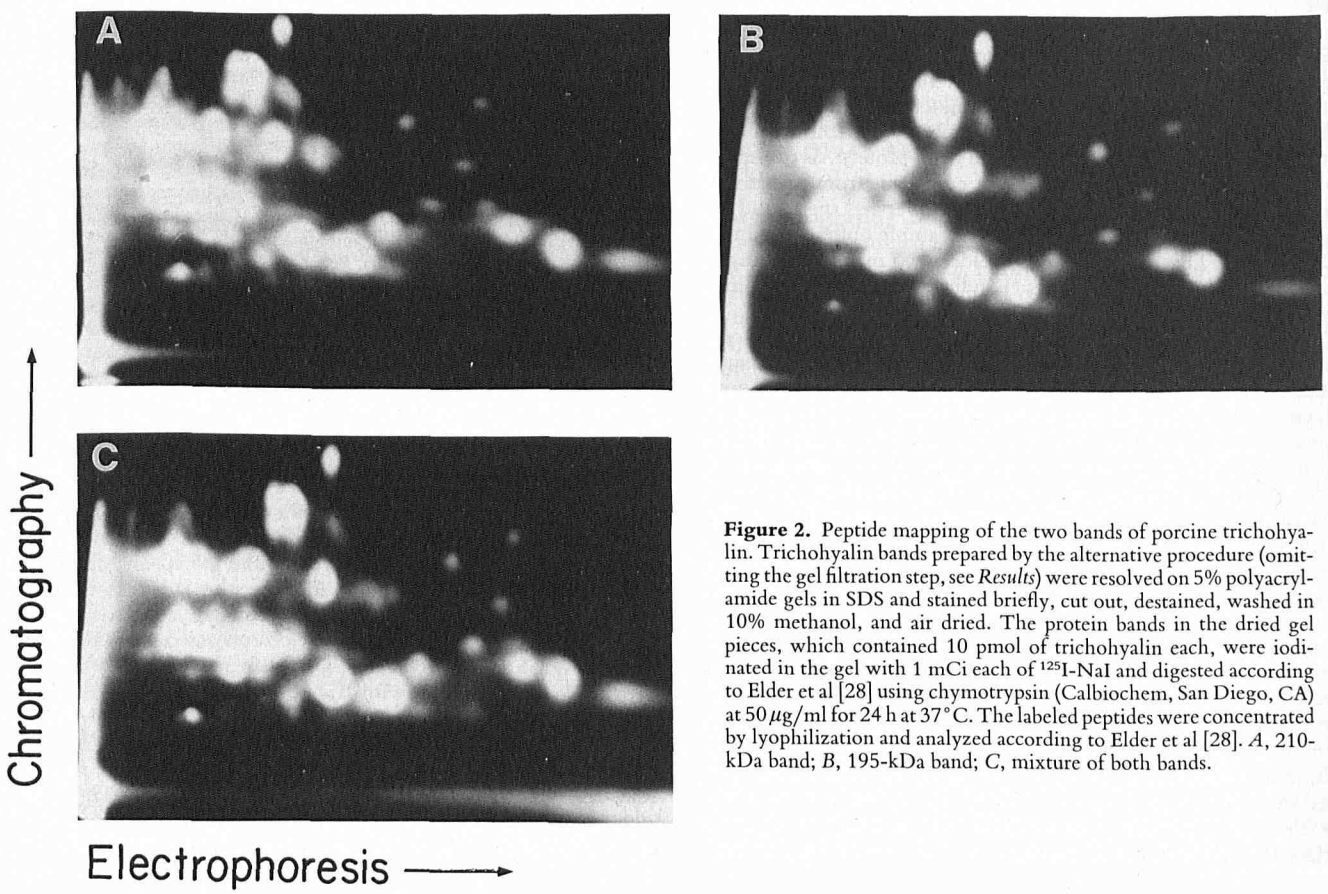

Figure 2. Peptide mapping of the two bands of porcine trichohyalin. Trichohyalin bands prepared by the alternative procedure (omitting the gel filtration step, see *Results*) were resolved on 5% polyacrylamide gels in SDS and stained briefly, cut out, destained, washed in 10% methanol, and air dried. The protein bands in the dried gel pieces, which contained 10 pmol of trichohyalin each, were iodinated in the gel with 1 mCi each of ^{125}I-NaI and digested according to Elder et al [28] using chymotrypsin (Calbiochem, San Diego, CA) at 50 μg/ml for 24 h at 37°C. The labeled peptides were concentrated by lyophilization and analyzed according to Elder et al [28]. *A*, 210-kDa band; *B*, 195-kDa band; *C*, mixture of both bands.

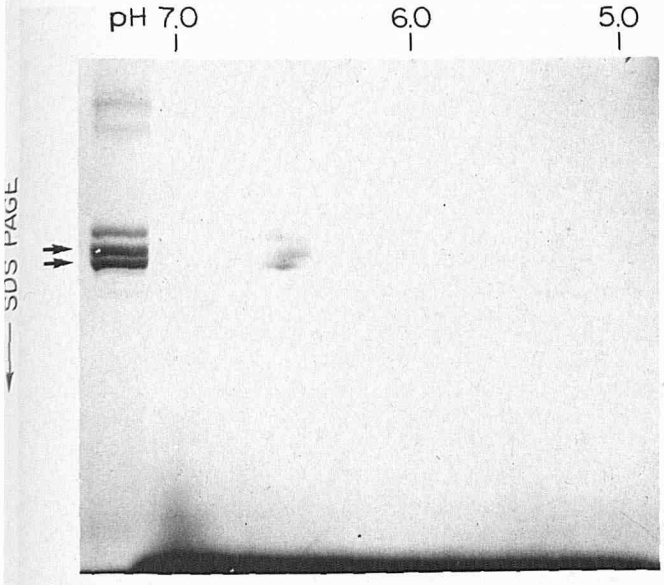

Figure 3. Isoelectric focussing of trichohyalin. Trichohyalin from the alternative procedure (see text), in which the supernatant from the ammonium sulfate precipitate (Fig 1, *lanes 4*) was resolved directly on a Mono Q column, was focussed as in *Materials and Methods*. One-dimensional SDS polyacrylamide electrophoresis of the sample is shown at the *left side* of the gel. (The highest M_r band was not observed in extracts of rapidly frozen epithelium, apparently developed during the purification, and reacted with antibody to trichohyalin.)

incubated with a crosslinking reagent in 1 M salt solution and then analyzed by SDS polyacrylamide gel electrophoresis and autoradiography. In the absence of crosslinker, the labeled protein had an M_r identical to the unlabeled material. Crosslinking of ^{125}I-labeled trichohyalin, however, produced high molecular weight species that failed to enter 3.5% polyacrylamide gels (not shown) or 0.5% agarose – 2% polyacrylamide gels (Fig 6) (in the presence of SDS in both cases), even though the composite gels should have been able to include oligomers of M_r as high as 1200–1400 kDa as determined by extrapolation from standards of M_r 46–205 kDa. The results indicate that under the conditions of the experiment, even at very low concentrations of protein possible using the radioactively labeled material (0.1 nm), the protein formed multimers when crosslinked. In order to reduce the tendency of trichohyalin to associate, we incubated the samples in urea and then added crosslinking reagent. When 4 M urea was included in the reaction, crosslinking produced only a single band of higher M_r than the monomer; this band had the M_r of a trichohyalin dimer, about 380 kDa (Fig 6). At higher concentrations of urea, no crosslinking occurred and only the monomer was detected on gels (not shown). In the presence of urea, the tendency of trichohyalin to associate into large multimers was reduced, revealing an intermediate stage in the association process, a dimer. Because the crosslinking reaction required an extended time (90 min) to produce a good signal on autoradiograms, it is likely that transient reversible associations were "captured" by the reaction, resulting in the production of large oligomers.

Table II. Physical Properties of Trichohyalin

R_s(nm)[a]	12.4
$s_{20,w}$[b]	6.0
Partial specific volume[c]	0.70
M_r, calculated[d]	282,000
f/f_o	2.3
M_r, SDS gel electrophoresis[e]	210,000/195,000

[a] Stokes radius, estimated from gel filtration (see *Experimental Procedures*).
[b] Sedimentation coefficient, estimated from sedimentation on 5–20% sucrose gradients [32].
[c] Estimated from amino acid composition [38].
[d] Calculations of M_r and frictional ratio were made according to the following equations [37]: $M_r = 6\pi N R_s s_{20,w}/(1 - v\rho 20,w)$, and $f/f_o = R_s[4\pi N/3M_r(\delta + \rho]^{1/3}$, with an assumed hydration of δ of 0.4 g/g of protein [39].
[e] Using buffer system of Fairbanks et al [27].

Table III. Amino Acid Composition of Porcine Trichohyalin

Asx	6.5 ± 0.5[a]
Glx	32.6 ± 1.3
Ser	4.6 ± 0.9
Gly	6.0 ± 0.6
His	<1
Arg	30.6 ± 1.2
Thr	<1
Ala	4.5 ± 0.2
Pro	1.7 ± 0.2
Tyr	<1
Val	1.8 ± 0.0
Met	<1
Cys	ND[b]
Ile	<1
Leu	8.1 ± 0.7
Phe	1.9 ± 0.1
Lys	2.1 ± 0.1
Trp	ND

[a] Mean ± SD of four determinations.
[b] ND, not done.

That the demonstration of an oligomer is based on true association of smaller species and is not a trivial artifact based on concentration alone is shown by the effect of 8 M urea, which, by interfering with intermolecular association, prevented crosslinking even though the protein concentration was the same. Therefore the ability of urea to reduce crosslinking indicates that some kind of equilibrium exists, such as monomer-monomer or dimer-dimer, and that higher order oligomers can also be formed.

The physical data shown in Table II show that a smaller species was present even at higher concentrations in 1 M salt, indicating that association into higher-order structures is very likely of low affinity and reversible. We concluded that trichohyalin can associate into higher-order structures but that the species of trichohyalin under study in most of our experiments (in 1 M salt) was a monomer or dimer (see *Discussion*).

Rotary Shadowing Electron Microscopy After elution from a Mono Q column as in Fig 1 (*lane 6*), trichohyalin was further purified on 15–40% glycerol gradients in 1 M ammonium bicarbonate and examined by electron microscopy as described in *Materials and Methods*. Rotary shadowed trichohyalin yielded images of a flexible molecule averaging 85 + 15 nm in length with a longer rod-shaped domain and a single globular-appearing end domain about 12 nm in diameter (Fig 7). Although there was heterogeneity in size of both the length of the rod and the diameter of the head, the molecules were consistent in appearance in having a distinct head and tail region. Because electron microscopy was performed in the same buffers or in the same ionic strength used to obtain the Stokes radius and Svedberg constant, it is likely that the form of the protein visualized by electron microscopy is the same upon which the physical measurements were made. As is noted above, the image probably represents a monomer or dimer of trichohyalin. It appeared that strands of the rod domain of two molecules were present in some of these images (Fig 7C), consistent with the possibility that the molecule isolated is a dimer.

DISCUSSION

Trichohyalin has been purified previously under denaturing conditions from sheep follicles by solubilization in 7 M guanidine, yielding a single 190-kDa polypeptide [2]. More recently a partial cDNA clone was obtained by these investigators [19]. Although amino acid composition of the extracted sheep wool follicle protein showed

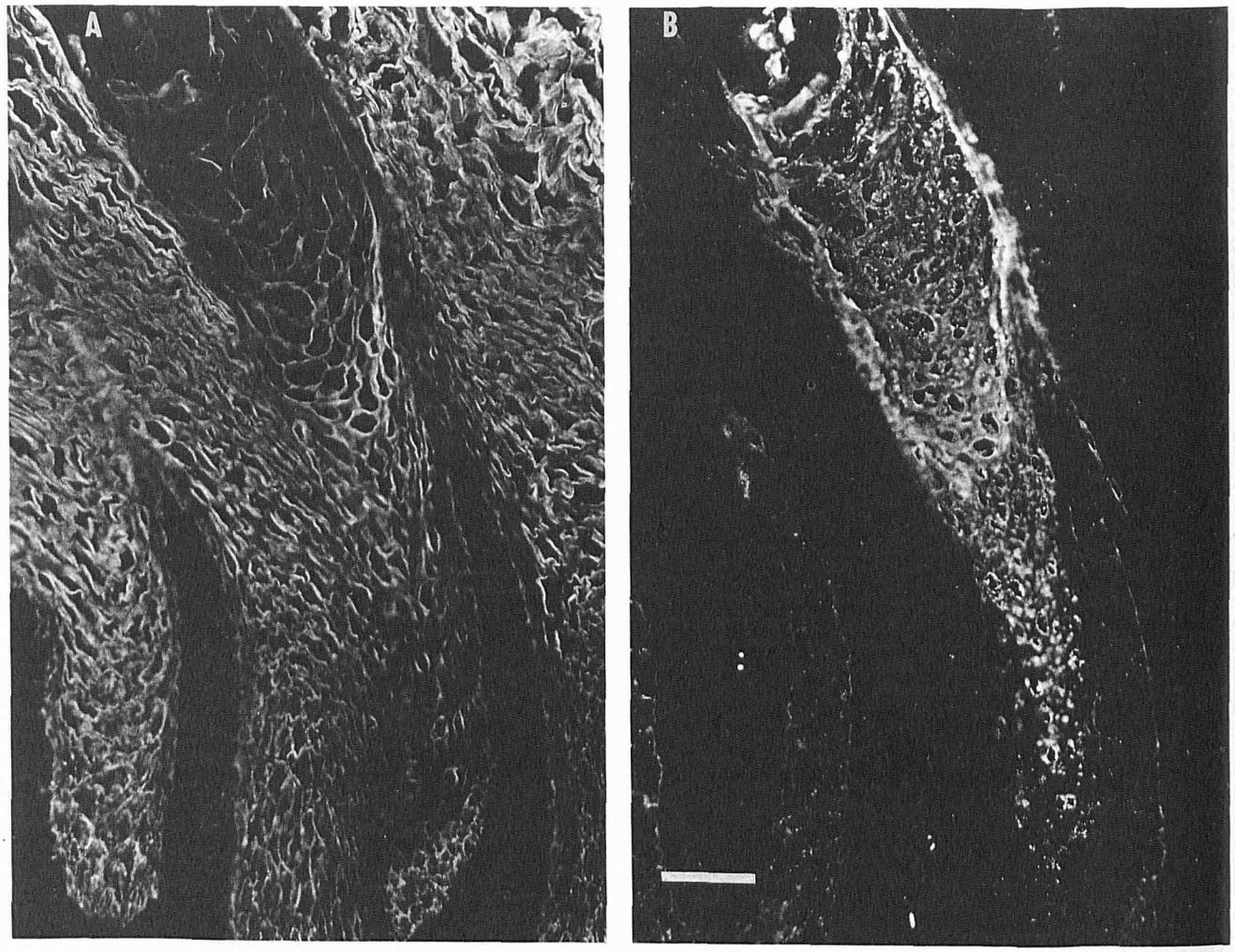

Figure 4. Demonstration of trichohyalin in filiform papillae. A single section of pig tongue epithelium was double-stained by indirect immunofluorescence as in *Methods* with affinity-purified rabbit antibody to desmoplakin I (*A*) or monoclonal antibody to trichohyalin (*B*). *Bar*, 70 μm.

only 13.7% arginine, the deduced structure indicated that arginine comprises about 24% of the cloned segment. This suggested to these authors that the carboxy-terminal third of the polypeptide differed from the more amino-terminal two-thirds [19]. Our analysis of the porcine protein (Table III) shows a high percentage of arginine, about 30%, comparable to that of the deduced fraction of the cloned segment of the wool follicle protein (24%), but much higher than that determined from purified wool follicle trichohyalin (14% [19]). Our results are consistent with the results of cDNA cloning of trichohyalin from sheep (G. Rogers, personal communication).

Immunofluorescence studies (Figs 4 and 5) indicate that trichohyalin is a major component of the filiform papilla of the tongue. These data, the present purification data, and the previously published immunoblots from tongue epithelium [25] demonstrate for the first time the presence and location of trichohyalin in the filiform papillae of the tongue. The relatively large amounts of trichohyalin in tongue permitted purification of the protein from a citric-acid insoluble fraction prepared according to a method devised initially by Skerrow and Matoltsy [36] to isolate a desmosome-rich fraction. Furthermore, as demonstrated by Coomassie blue staining and immunoblotting of various fractions during our purification studies, most of the trichohyalin in the tongue epithelium was lost in association with the keratin filaments solubilized in citric-acid buffer. When citric acid–solubilized keratins from pig tongue epithelium were made insoluble, either a) by dialysis against 4 M urea and then against low ionic strength neutral buffers that caused assembly of keratin filaments or b) by isoelectric precipitation produced by rapid neutralization of the citrate buffer, trichohyalin coprecipitated quantitatively with keratins. Although low ionic strength and increased temperature released trichohyalin from the citric acid–insoluble pellet as described above (Fig 1), this treatment did not release trichohyalin from the isoelectric precipitate or from keratin filaments polymerized under conditions of low ionic strength (5 mM sodium phosphate) at neutral pH (not shown). Therefore we were unable to separate trichohyalin from the fraction containing almost exclusively keratin. These findings do not distinguish between the two possibilities that trichohyalin either a) was physically associated with keratin filaments or b) was not associated but had similar solubility properties and copurified with keratins for this reason alone. Nevertheless, the ability of trichohyalin to remain associated with keratins through repeated cycles of assembly suggests that the two proteins may be physiologically associated through a specific binding interaction. When keratin-associated trichohyalin was resolved by gel filtration in urea, the amount of trichohyalin purified was small relative to keratin, so that this procedure was not useful for preparative work.

Trichohyalin has the mobility on SDS gels of a monomer, in agreement with previously published M_r. The data for sedimentation constant and Stokes radius as well as electron microscopic images were obtained in 1 M salt solutions required to maintain

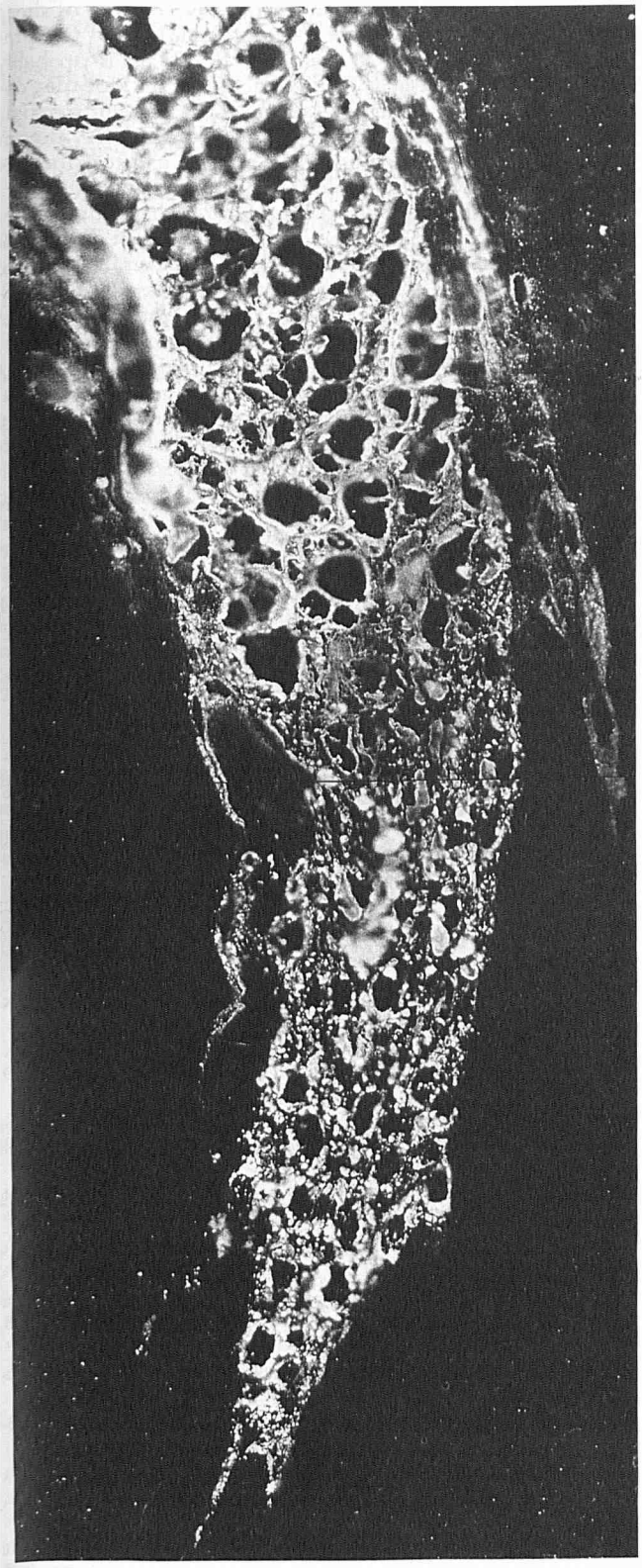

Figure 5. Granular to amorphous progression of trichohyalin in a tongue papilla. Sections of pig tongue epithelium were stained by indirect immunofluorescence as in *Methods* with affinity-purified rabbit antibody to trichohyalin. A montage including most of the length of a filiform papilla demonstrates staining of small granules at the base of the papilla, larger granules in the mid-region, and amorphous cellular staining at the most mature region. *Bar*, 50 μm.

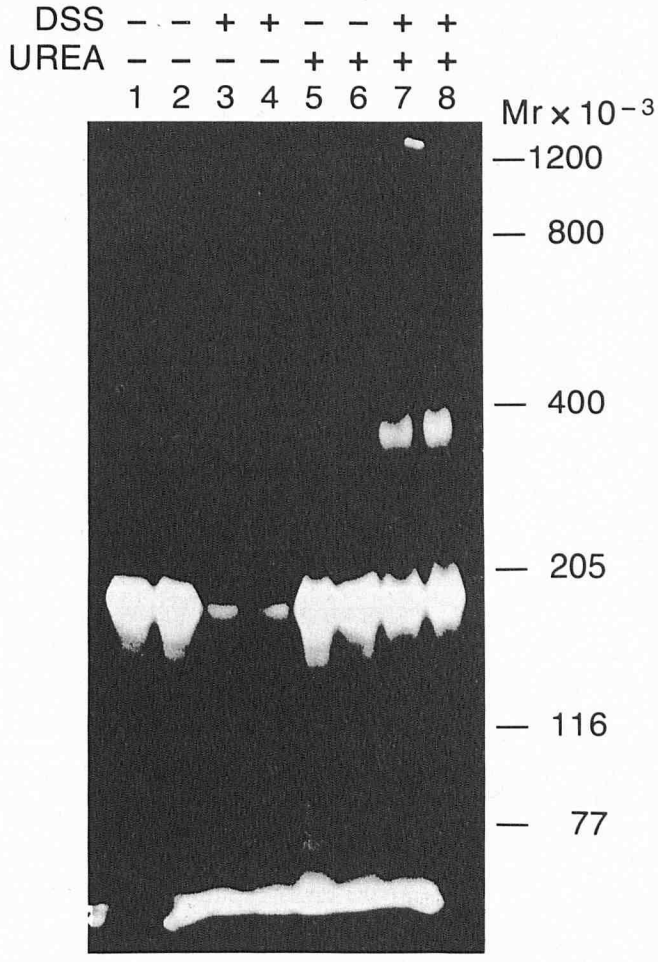

Figure 6. Crosslinking of trichohyalin. Trichohyalin derivatized with ^{125}I-labeled Bolton-Hunter reagent (2×10^5 cpm) was incubated with or without the bifunctional crosslinker, disuccinimidyl suberate (0. 8 mM), in buffers containing 1 M NaBr (*lanes 1–4*) or 4 M urea (*lanes 5–8*) in 10 mM sodium phosphate buffer, pH 8, containing 1 mM EDTA, 1 mM dithiothreitol, and 0.05% Tween-20 for 90 min on ice. The reaction was stopped by addition of glycine (2 mM final concentration), and the samples were analyzed by SDS agarose-polyacrylamide gel electrophoresis and autoradiography.

trichohyalin in solution and demonstrate that the molecule under these conditions is extended and asymmetric ($f/f_0 = 2.3$, Table II) and has a molecular weight calculated according to Tanford [37] from these data of about 282,000 Da. Because this is 1.4 times the M_r of the monomer by SDS gel electrophoresis, it is possible that the form of the protein being studied in 1 M salt solutions is not the monomer but a dimer, or there may be an equilibrium between monomer and dimer. It is unlikely, however, that the species in 1 M salt is larger than a dimer. It order to test for the presence of a dimer directly, we performed crosslinking experiments.

We used urea to reduce the strength of intermolecular association and hence reduce the capture of transient oligomeric associations during the prolonged crosslinking reaction. At a urea concentration of 4 M, it was possible to demonstrate the presence of trichohyalin dimers. This does not establish that the species under study in Table II and in electron microscopy is a dimer but is compatible with the possibility that the calculated molecular weight, which is intermediate between a monomer and dimer according to SDS gels, reflects the presence of a dimer or a monomer-dimer equilibrium in 1 M salt solutions. The electron microscopic images are compatible with the presence of a dimer but do not exclude other possibilities. The M_r

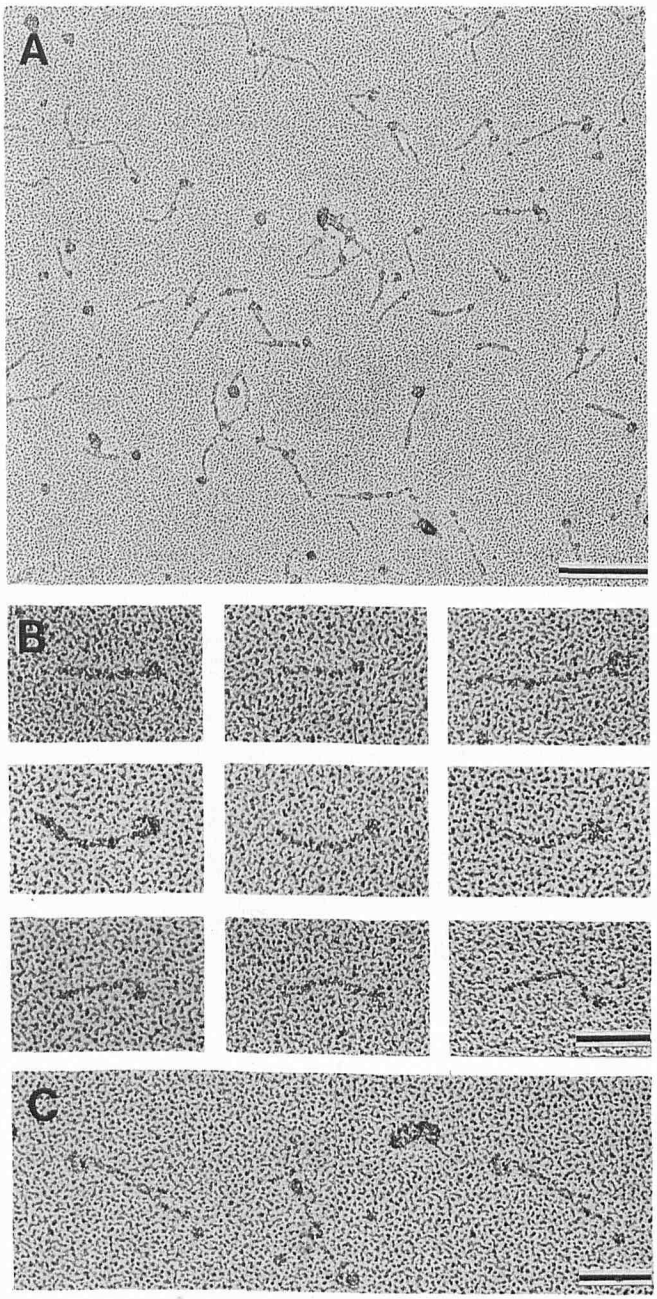

Figure 7. Rotary shadowing electron microscopy. Purified trichohyalin from the Mono Q column was collected from a 15–45% glycerol gradient centrifugation, (performed as for sucrose gradients in *Materials and Methods*), and diluted and processed as in *Materials and Methods*. *A*, a representative field. *Bar*, 100 nm. *B*, representative molecules, showing variation in length and characteristic structure. *Bar*, 50 nm. *C*, a stereomicroscopic view of a molecule showing the apparent double-stranded quality of the isolated molecules. *Bar*, 50 nm.

determined by SDS polyacrylamide gel electrophoresis may overestimate the true molecular weight of the monomer; in this case the derived value for trichohyalin in solution may be approximately two times the molecular weight of the monomer. This possibility will be resolved by determining the molecular weight of trichohyalin by cDNA cloning.

Because of this uncertainty, we are unable to determine at present whether the structure visualized by electron microscopy is a monomer or dimer. Fietz et al [19] have shown by cDNA cloning of sheep wool follicle trichohyalin that the C-terminal one third of the protein is composed of tandem 28 amino-acid repeats and is probably capable of forming an α-helix. This suggests that the globular domain resides in the N-terminus. Additional electron microscopic studies, for example of proteolytic fragments of trichohyalin, as well as data from cDNA cloning will be required to determine the structure of trichohyalin in the electron microscopic image.

How trichohyalin might associate to form higher-order structures in vivo is not clear from our studies. We did not find what appeared on electron microscopy to be larger oligomers in a stacked side-to-side association of the dimers, but more concentrated specimens, or trichohyalin in the presence of another protein capable of mediating higher-order assembly, may associate in some other fashion such as by head-to-head or head-to-tail association. The correct molecular weight and information about possible modes of association of monomers may be provided by elucidation of the complete primary structure. The trichohyalin preparation shown here results in native trichohyalin to the extent that the protein assumes an ordered structure in solution, as documented by physical studies and electron microscopy, and does not aggregate. The studies reported here provide a basis for functional studies complementing the cloning data using native trichohyalin, for example, to determine whether it can bind directly to keratin filaments, whether affinity for keratin is limited to specific types of keratin found associated with trichohyalin in the inner root sheath or filiform papilla, and ultimately the location of the presumed binding sites on trichohyalin and keratin.

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